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Salmonella typhimurium Mutants Lacking Flagella or Motility Remain Virulent in BALB/c Mice

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Nonmotile flagellated (*mot*) and nonflagellated (*fla*) mutants of *Salmonella typhimurium* LT-2 were isolated from a collection of mutants with random Tn*10*-insertion mutations. Both classes of mutants were resistant to infection by the flagellotropic bacteriophage χ. The nonflagellated (*fla*::Tn*10*) mutants did not react with H antigen-specific antisera and did not possess flagella when examined by electron microscopy, and sheared-cell extracts were devoid of flagellin. The nonmotile (*mot*::Tn*10*) mutants reacted with H-specific antisera and expressed paralyzed flagella that were indistinguishable from wild-type flagella. The Tn*10* insertions in strain LT-2 were mapped to loci in regions II (*flh* and *mot*) and III (*fli*) of the flagellar genes, and the mutations were transduced into the mouse-virulent *S. typhimurium* strains SR-11 and SL1344. Lack of motility reduced the ability of *S. typhimurium* to invade Henle cells in vitro, yet the virulence in mice of the nonmotile mutants of SR-11 and SL1344 was unaffected by the inactivity or loss of flagella. Wild-type SR-11 had a 50% lethal dose (LD_{50}) in BALB/c mice following oral (p.o.) challenge of 2.4×10^4 CFU. The p.o. LD_{50} of the SR-11 *fli-8007*::Tn*10* mutant was 4.5×10^4 CFU. The *mot-8008*::Tn*10* mutation in SR-11 conferred paralyzed flagella and increased the p.o. LD_{50} in mice to 2.2×10^5 CFU, but this was not statistically significant. A similar increase in the p.o. LD_{50} was observed when the SL1344 *mot-8008*::Tn*10* mutant was tested in mice. Wild-type SR-11 and the isogenic nonflagellated and nonmotile mutants were equally virulent in mice challenged via intraperitoneal injection.

Flagella (H antigen) on the surface of *Salmonella typhimurium* have been characterized as virulence factors that help the bacteria move toward and adhere to host cells. Several groups of investigators have studied *S. typhimurium* rendered immobile by mutation or by the use of antiflagellar antibodies to demonstrate decreased invasiveness for mammalian cells cultured in vitro. Jones et al. (26) reported that nonflagellated (*fla*) mutants derived from virulent strains of *S. typhimurium* exhibited decreased ability to adhere to and invade HeLa cells, while Tomita and Kanegasaki (48) found that a nonmotile, yet flagellated (*mot*), *S. typhimurium* mutant was less invasive in macrophages. In both cases, invasion of mammalian cells by nonmotile bacteria was enhanced by gently centrifuging the bacteria onto the tissue culture monolayers. Fields et al. (14) also isolated Tn*10*-induced nonmotile mutants of *S. typhimurium* that were less able to invade and survive in macrophage cultures in vitro. Recently, Liu et al. (32) described the isolation of *Salmonella typhi fla* and *mot* mutants that displayed decreased invasiveness for mammalian cells in culture. In this case, however, adherence of the bacteria to the mammalian cells was unchanged by mutations affecting motility, and invasiveness was not augmented by centrifugation of bacteria onto monolayers. Invasiveness of wild-type *S. typhi* was also inhibited by antiflagellar sera (32).

Fewer studies on the pathogenesis of nonmotile *S. typhimurium* in mice have been reported. Passive immunization of mice with anti-H antiserum did not protect the animals from a lethal challenge with virulent organisms, although the antiserum inhibited bacterial adherence to intestinal epithelium in vitro (19). Hackett et al. (18) used isogenic *fla*$^+$ or *fla* *Salmonella* strains to investigate the contribution of flagella in immunizing mice against infection by *S. typhimurium*. Mice vaccinated orally with flagellated salmonellae survived longer than mice that received *fla* mutants after subsequent oral challenge with $>10^3$ times the 50% lethal dose (LD_{50}) of a motile, virulent strain of *S. typhimurium*. Nonflagellated strains colonized the intestinal tracts of orally vaccinated mice as well as isogenic flagellated strains yet did not confer equal protection from subsequent lethal challenge by motile *S. typhimurium* (18). Carsiotis et al. (8) and Weinstein et al. (49) compared wild-type motile *S. typhimurium* and derivatives made nonmotile by the introduction of specific mutations affecting motility (*motB*) or flagellar biosynthesis (*flaF25*). Their data indicated that the presence of flagella on the surface of wild-type or *mot* cells aided bacterial invasiveness and survival in macrophages and increased outright virulence for mice. The *flaF25* allele was originally described as a deletion of unknown size within the *flaF* gene cluster (23) but was subsequently reported as a deletion of genes *flaFI* through *flaFV* (28). Recently, Carsiotis et al. (M. Carsiotis, B. A. D. Stocker, I. A. Holder, D. Weinstein, and A. D. O'Brien, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, B-169, p. 53) reported that the mutation involved not only some of the genes encoding the biosynthesis of flagella but extended into a previously undescribed virulence gene(s). Thus, the role of the *flaF25* mutation in the attenuation of *S. typhimurium* in mice remained unclear. (The flagellar genes of *S. typhimurium* and *Escherichia coli* have recently been renamed [24], and *flaFI* through *flaFV* are now known as *flgA* through *flgE* of region I. In the present report the original nomenclature is retained in discussions of the studies conducted by Carsiotis et al. [8] and Weinstein et al. [49] while the recently revised genotypic designations are used to describe the mutations that were identified during the work detailed below.)

Invasion of in vitro-cultured tissues by *S. typhimurium* permits the study of an important phase of virulence, namely, the spread of the pathogen beyond the intestinal tract. However, results obtained in vitro (e.g., adherence to

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TABLE 1. Bacterial strains

Strain	Genotype	Source
LT-2	Wild type	51
SR-11	Wild type, P22^i	34, 45
SL1344	*hisG rpsL xyl*	20
χ3370	*fli-8001*::Tn*10* in LT-2	This work
χ3373	*flh-8004*::Tn*10* in LT-2	This work
χ3376	*fli-8007*::Tn*10* in LT-2	This work
χ3377	*mot-8008*::Tn*10* in LT-2	This work
χ3419	*mot-8008*::Tn*10* derivative of SR-11 transduced by P22 grown on χ3377	This work
χ3420	*fli-8007*::Tn*10 hisG rpsL xyl* derivative of SL1344 transduced by P22 grown on χ3376	This work
χ3421	*mot-8008*::Tn*10 hisG rpsL xyl* derivative of SL1344 transduced by P22 grown on χ3377	This work
χ3422	*fli-8007*::Tn*10* derivative of SR-11 transduced by P22 grown on χ3376	This work
KK1011	Δ(*flgA-flgL*) Δ(*fljAB-hin*)	29
KK1012	Δ(*flhA-motBA-flh*) Δ(*fljAB-hin*)	29
KK1013	Δ(*fliA-fliR*) Δ(*fljAB-hin*)	29
SJW1368	*hsdL6* Δ(*cheW-motBA-flhCD*) *hisC27 rpsL120 xyl-404 ilv-452 metE55 metA22 hsdSA29*	28
SJW1399	*hsdL6* Δ(*flhAB-cheA*) *hisC527 rpsL120 xyl-404 ilv-452 metE55 metA22 hsdSA29*	28
SJW1411	Δ(*fliA-fliD*)	21, 27, 50
SJW1606	Δ(*fliG-fliR*)	50
SJW1682	Δ(*fliA-fliI*)	50

and invasion of mammalian cells was blocked by H-specific antibodies [18, 32]) might not extend to events in vivo (e.g., mice passively immunized with H-specific antibodies were not protected from infection by *S. typhimurium* [18]). The use of the murine model of typhoid fever provides a critical evaluation of putative virulence factors in the development of disease. This report describes the isolation of nonmotile mutants of *S. typhimurium* and characterization to determine that the mutations were limited to genes controlling flagellum biogenesis or movement. When the mutations were transferred to mouse-virulent salmonellae, no significant changes in virulence for mice were observed.

MATERIALS AND METHODS

Bacteria, bacteriophages, and media. The strains of *S. typhimurium* that were studied are listed in Table 1. The Tn*10* mutant library in strain LT-2 has been described previously (10). In most cases bacteria were grown in L broth (31) and the medium was solidified by the addition of 1.5% agar, or, when necessary, 0.5% agar for motility tests and phage work (see below). The minimal medium used in these experiments was that of Curtiss (9), and 22 μg of L-histidine per ml was included for growth of strain SL1344 and its derivatives. Strains containing Tn*10* were grown in the presence of tetracycline (25 μg/ml), and for phage work the media were supplemented with 2.5 mM $CaCl_2$. Phage χ recognizes functional flagella as receptors (40) and was obtained from K. E. Sanderson. P22HT *int* (44) was used to effect transduction between bacterial strains (11). Incubations were at 37°C unless stated otherwise.

Motility tests. Hanging-drop preparations were examined by phase-contrast microscopy. Cultures were also grown in pour plates of semisolid agar or by stab inoculating petri dishes containing semisolid medium. Plates were incubated for up to 72 h.

Serology. Antisera specific for Kauffmann and White factor i and single factor 2 were purchased from Difco Laboratories (Detroit, Mich.), and agglutination tests were performed according to the directions of the manufacturer.

Phage sensitivity. Sensitivity to P22HT *int* was assessed by cross-streaking bacterial cultures and phage lysates or by plating mixtures of bacteria and phage in semisolid agar to obtain individual plaques. Phage χ sensitivity was determined by spotting lysates on semisolid agar seeded with bacterial cells or by plating to obtain isolated plaques. All plates were incubated for 18 to 24 h.

Plasmid analysis. Cultures were extracted by the method of Birnboim and Doly (6), and extracts were examined after electrophoresis in 0.7% agarose gels prepared in Tris-borate–EDTA buffer (39).

Surface hydrophobicity. The salt aggregation test and bacterial adhesion to hydrocarbons were done as described by Smyth (47).

Analysis of flagellin. Sheared-cell extracts were prepared from cells grown overnight without agitation in shallow broth cultures. Cells were pelleted by centrifugation and then suspended in cold buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 0.01% NaN_3). Flagella were sheared from the cells in a Brinkman PT10/35 homogenizer fitted with a PTA10TS generator and operated at a dial setting of 5. Cells were homogenized on ice in cycles of 30 s on–30 s off for a total of 2 min, and the bacteria were then removed by centrifugation. The amount of total protein in the extracts was determined by using a commercially available bicinchoninic acid assay (Pierce Chemical Co., Rockford, Ill.). Extracts were examined directly or after collecting the precipitate formed from adding $(NH_4)_2SO_4$ to 50% of saturation. Samples were dissociated in sample buffer at 100°C for 10 min and analyzed by electrophoresis on 8% polyacrylamide gels in the presence of sodium dodecyl sulfate (33). Gels were stained with Coomassie blue R250.

Electron microscopy. Glutaraldehyde-fixed bacterial cultures were applied to carbon-coated films of Butvar-98 on 300-mesh copper grids, negative stained with 2% (wt/vol) uranyl acetate, and rinsed with 0.2% uranyl acetate. Specimens were viewed in a Hitachi H-600 transmission electron microscope at 75 kV.

Genetic mapping. Individual strains of *S. typhimurium* with well-characterized deletions of different *fla* genes (21, 27–29, 50) were obtained from Robert Macnab (Yale University, New Haven, Conn.) and from Kazuhiro Kutsukake (Tokyo University, Tokyo, Japan). Recombinational analysis was performed by P22-mediated transduction between strains with known mutations and the strains described in this report.

Animal studies. Six- to eight-week old female BALB/c mice were used in all experiments. All bacterial strains were passaged once in mice prior to determination of the LD_{50}. Animal passage consisted of feeding 1×10^7 to 2×10^7 bacteria to mice and reisolating the challenge organisms from splenic homogenates 3 days postchallenge. Animal inoculations for the determination of the LD_{50} values were performed essentially as described by Gulig and Curtiss (17). The oral challenge regimen was modified by withholding food from the mice overnight and then removing the drinking water for 4 to 5 h prior to oral challenge. Gastric acidity in mice was neutralized by administering orally (p.o.) 50 μl of a solution of 1% (wt/vol) $NaHCO_3$. LD_{50} titers were calculated by the method of Reed and Muench (42) from results obtained from five mice per inoculum dose.

TABLE 2. Characterization of nonmotile *S. typhimurium* strains

Strain	Motility	Agglutination by H antisera	Sensitivity to bacteriophage[a]: P22	Sensitivity to bacteriophage[a]: χ	Growth on minimal glucose medium[b]	100-kb[c] virulence plasmid
LT-2	+	+	S	S	+	+
SR-11	+	+	R	S	+	+
SL1344	+	+	S	S	+	+
χ3376	−	−	S	R	+	+
χ3377	−	+	S	R	+	+
χ3419	−	+	R	R	+	+
χ3420	−	−	S	R	+	+
χ3421	−	+	S	R	+	+
χ3422	−	−	R	R	+	+

[a] S, Sensitive; R, resistant.
[b] Medium was supplemented with histidine for SL1344 and its derivatives.
[c] kb, Kilobase.

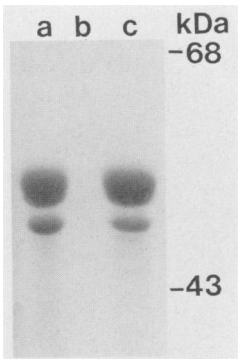

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis comparison of flagellin from wild-type and nonmotile strains of *S. typhimurium*. Lane a, SR-11 (Fla$^+$ Mot$^+$); lane b, χ3422 (Fla$^-$ Mot$^-$); lane c, χ3419 (Fla$^+$ Mot$^-$). kDa, Kilodaltons.

In vitro invasion assay. Henle-407 cells were used in tissue culture as previously described (15, 17).

RESULTS

Isolation of nonmotile mutants. Samples of a culture of *S. typhimurium* LT-2 containing random Tn*10* insertions were pour plated in semisolid medium containing tetracycline, and after incubation at 37°C, colonies that did not exhibit expanding zones of growth were isolated. Stable nonmotile mutants of LT-2 represented 0.05% of the tetracycline-resistant (Tet^r) cells in the library. Nonmotile mutants were examined serologically and by light microscopy after staining for flagella (37) in order to distinguish mutants with inactive flagella from those lacking flagella entirely. χ3376 and χ3377 (Table 1) were initially chosen as representative nonflagellated (*fli-8007*::Tn*10*) and nonmotile (*mot-8008*::Tn*10*) mutants and were analyzed further. P22HT *int* grown on either χ3376 or χ3377 was used to transduce strains SR-11 and SL1344 to Tet^r. All Tet^r transductants were also nonmotile, and χ3419, χ3420, χ3421, and χ3422 were saved as nonmotile mutants of mouse-virulent salmonellae (Table 1).

Comparison of wild-type and nonmotile *S. typhimurium*. Wild-type *S. typhimurium* strains were highly motile and rapidly produced radiating areas of growth after inoculation by stabbing into semisolid medium. χ3376, χ3377, and χ3419 through χ3422, in contrast, remained at the site of inoculation even after 72 h of incubation (Table 2). The nonmotile mutants were characterized serologically with H-specific antisera. The agglutination reactions of χ3377, χ3419, and χ3421 were as strong as the reactions of the motile parental strains. χ3377, χ3419, and χ3421 reacted equally well with either factor i H antiserum or with single factor 2 H antiserum, indicating that these mutants expressed both flagellar-phase antigens. χ3376, χ3420, and χ3422 were not agglutinated by H-specific antisera. All of the nonmotile mutants were resistant to infection by phage χ, which requires the presence of functional flagella to infect *S. typhimurium* (40). The nonmotility conferred by Tn*10* insertions was not an indirect pleiotropic effect of mutations that altered lipopolysaccharide biosynthesis (1), since χ3376, χ3377, χ3420, and χ3421 remained sensitive to phage P22. Additional evidence that the nonmotile mutants were not defective in some nonspecific aspect of assembly or function of extracellular filaments was provided by the normal expression of type 1 pili by the nonmotile strains of *S. typhimurium* (data not shown).

All nonmotile *S. typhimurium* strains were able to grow on minimal medium containing glucose (Table 2), indicating that auxotrophic requirements were not developed during the construction of the strains. The plasmid content of these strains was also examined, and all strains contained the 100-kilobase virulence plasmid that has been shown to be necessary for *S. typhimurium* virulence (Table 2) (17).

Cell surface hydrophobicity was measured by two alternate methods. Aggregation of motile and nonmotile bacteria occurred at an $(NH_4)_2SO_4$ concentration of 4.0 M, and none of the wild-type or mutant strains demonstrated significant adherence to *n*-hexadecane or *p*-xylene. These assays indicated that no change in surface hydrophobicity accompanied the loss of motility or flagella.

Sheared-cell extracts prepared from strains SR-11 and χ3419 contained equal quantities of protein, present largely as flagella and pili. Extracts from flagellated strains SR-11 and χ3419 contained about 30 times more total protein than extracts prepared from χ3422. Figure 1 shows a portion of an 8% polyacrylamide gel which displays flagellar proteins in these extracts. The proteins with the masses 53 and 49 kilodaltons in lanes a and c are equivalent to the flagellar antigens expressed by *S. typhimurium* (36, 46). Samples of extracts from SR-11 (Fig. 1, lane a) and χ3419 (lane c) were identical in this analysis. A similarly prepared sheared-cell extract from χ3422 is shown in Fig. 1, lane b. This lane was loaded with approximately 12 times more extract than was loaded in the adjacent lanes and did not contain detectable flagellin. Concentrated supernatant fluids from cultures of χ3422 also did not contain flagellar proteins.

Wild-type and mutant *S. typhimurium* strains were examined by electron microscopy (Fig. 2). The number of flagellar filaments per cell and the filament morphology were indistinguishable between wild-type strain SR-11 and the *mot-8008*::Tn*10* mutant, χ3419 (Fig. 2a and b). No flagella were observed on cells of the *fli-8007*::Tn*10* mutant, χ3422 (Fig. 2c).

Genetic mapping of mutations conferring nonmotility. P22HT *int* was propagated on χ3376 containing the *fli-8007*::Tn*10* allele and used to infect strains KK1011, KK1012, and KK1013, each with a different deletion of one of the regions involved in flagellar biosynthesis, with selection for transductants exhibiting motility. Motile transductants were obtained with KK1011 (Δ*flgA-flgL*) and KK1012 (Δ*flhA-motBA-flhD*) but not with KK1013 (Δ*fliA-fliR*). Transduction of strain SJW1411 with P22HT *int* grown on χ3376 yielded motile transductants, but no motile transductants

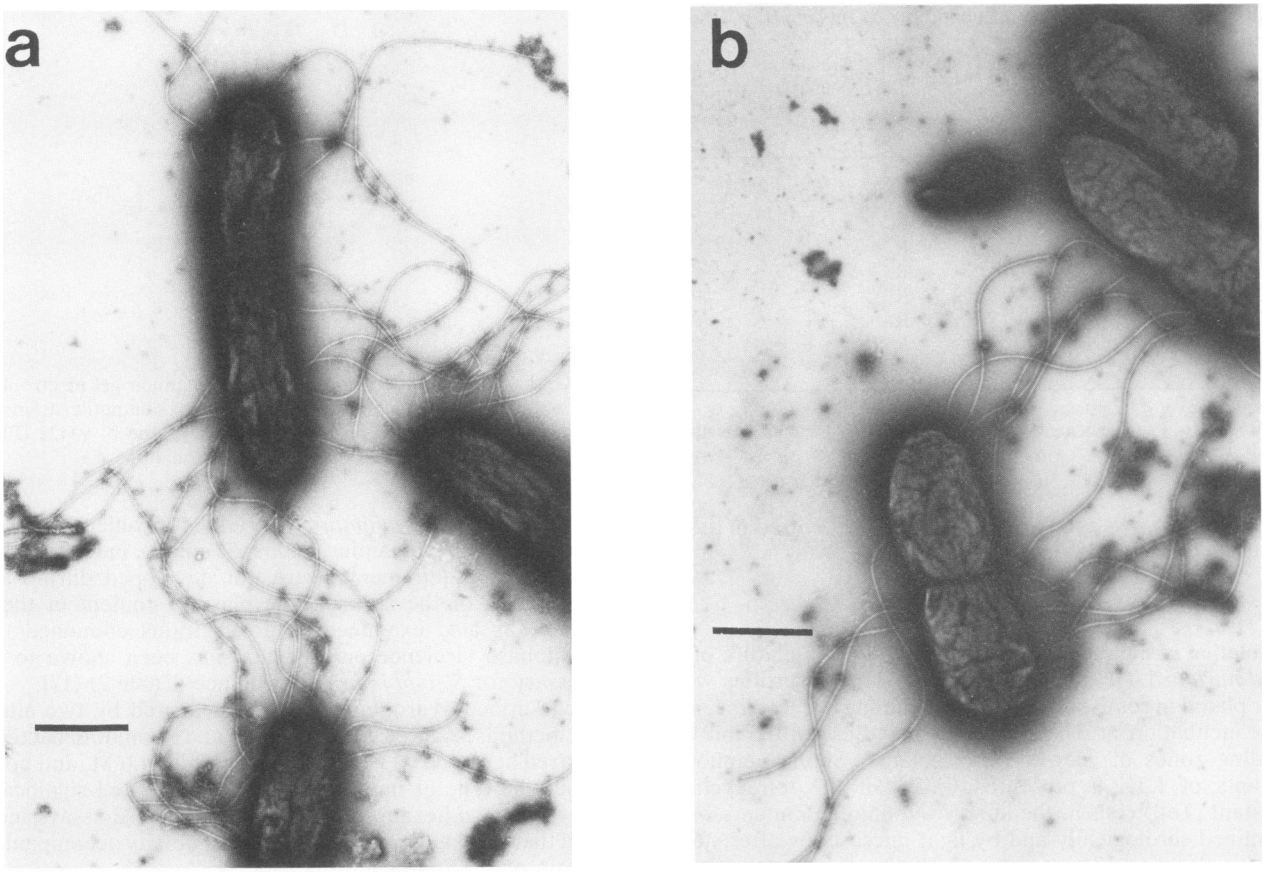

FIG. 2. Electron micrographs of wild-type and nonmotile strains of *S. typhimurium*. (a) SR-11 (Fla^+ Mot^+); (b) χ3419 (Fla^+ Mot^-); (c) χ3422 (Fla^- Mot^-). Bars, 1 μm.

were obtained from transductions of strains SJW1606 and SJW1682. The location of the Tn*10* insertion mutation in χ3376 was thus limited to the area of overlap of the deletions in SJW1606 and SJW1682, which involved *fliGHI*. Transduction of strains KK1011, KK1012, and KK1013 with P22HT *int* propagated on χ3377 containing the *mot-8008*::Tn*10* allele yielded motile transductants with strains KK1011 and KK1013 but not with KK1012. Transduction of strain SJW1399 with P22HT *int* grown on χ3377 produced motile transductants, but no motile transductants were obtained from transduction of strain SJW1368. The Tn*10* insertion in χ3377 resulted in nonmotile cells that were resistant to phage χ, yet possessed flagella, thus limiting the location of the insertion to *motBA*. Analysis of additional nonmotile mutants isolated from the Tn*10* insertion library in LT-2 did not yield mutations that were mapped to region I of the flagellar genes. Insertions were identified that were distinguishable from *fli-8007*::Tn*10*, however (Table 1). *fli-8001*::Tn*10* was mapped to the area of *fliJ* through *fliR*, and *flh-8004*::Tn*10* was located in *flhCD*.

Virulence of nonmotile mutants. Wild-type and *fli-8007*::Tn*10* strains of *S. typhimurium* were equally virulent for mice challenged p.o. (Table 3). The p.o. LD_{50} of strain SR-11 was 2.6×10^4 CFU, and that of χ3422 was 4.5×10^4 CFU. Oral challenges of mice with the paralyzed *mot-8008*::Tn*10* mutant, χ3419, demonstrated a slightly elevated LD_{50} of 2.2×10^5 CFU. Wild-type strain SL1344 was slightly less virulent than SR-11 by the p.o. route, but the effect of introducing either the *fli-8007*::Tn*10* or *mot-8008*::Tn*10* mutation was the same. SL1344 and the *fli-8007*::Tn*10* mutant, χ3420, displayed similar p.o. LD_{50}s, whereas the *mot-8008*::Tn*10* mutant, χ3421, had a slightly increased p.o. LD_{50} (Table 3). The difference between the p.o. LD_{50}s for the wild-type and *mot-8008*::Tn*10* strains, while reproducible, was not statistically significant when compared in a two-tailed Student's *t* test. Wild-type *S. typhimurium* strain SR-11 and both nonmotile derivatives of SR-11 were highly

TABLE 3. Virulence of nonmotile *S. typhimurium* strains

Strain	Phenotype	LD_{50} (CFU)		Mean no. of days to death[a]	Invasion of Henle cells[b]
		p.o.	i.p.		
SR-11	Fla^+ Mot^+	2.6×10^4	2	11	57 ± 0.2
χ3419	Fla^+ Mot^-	2.2×10^5	4	8	4.5 ± 0.02
χ3422	Fla^- Mot^-	4.5×10^4	1	10	7.3 ± 0.04
SL1344	Fla^+ Mot^+	1.3×10^5	ND[c]	10	ND
χ3420	Fla^- Mot^-	5.0×10^5	ND	10	ND
χ3421	Fla^+ Mot^-	1.1×10^6	ND	11	ND

[a] Data taken 30 days postchallenge for mice receiving approximately 1 LD_{50} p.o.

[b] Mean percentage ± standard deviation of CFU recovered from triplicate wells in duplicate experiments.

[c] ND, Not determined.

virulent for mice challenged intraperitoneally (i.p.) (Table 3), and there were no significant differences between the i.p. LD_{50}s for any of the strains. Mice infected by the same route displayed similar average times to death regardless of the challenge strain. Several alternative mutations were tested to determine whether the virulence of the nonmotile strains was related to the location of the Tn*10* insertion. The virulence of SR-11 in mice was unaffected by the introduction of *fli-8001*::Tn*10* or *flh-8004*::Tn*10* (Table 1) (data not shown).

The possibility that the nonmotile mutants reverted to wild type in vivo was examined in parallel with the LD_{50} determinations. Additional animals were challenged with 10^8 CFU p.o. or with 500 CFU i.p. At intervals over the course of the LD_{50} experiments a fraction of these animals was sacrificed and their spleens were homogenized and plated on nonselective media to recover the challenge strains. Bacterial isolates were then tested for motility. All of the *S. typhimurium* organisms isolated from infected spleens had the same motility phenotype as the respective challenge organisms. χ3419 reisolated from mice was agglutinated by anti-H antisera, and χ3422 recovered from infected mice was nonagglutinating.

The nonmotile derivatives of SR-11 were less efficient at invading Henle cells in vitro (Table 3). Fifty-seven percent of the SR-11 CFU that were applied to monolayers was recovered after 3 h of incubation to permit invasion and 3 h of additional incubation in the presence of gentamicin to select intracellular bacteria. Under the same conditions, 4.5% of the χ3419 CFU and 7.3% of the χ3422 CFU that were inoculated were recovered.

DISCUSSION

Flagella enable bacterial cells to move chemotactically in response to stimuli (35, 36), and there is evidence that these organelles function in the attachment of certain bacteria to solid surfaces (2, 4, 16, 19, 25, 26). Although many enteric pathogenic bacteria are motile, possession of flagella is not a requisite for virulence in all cases. Species of the genus *Shigella* are uniformly nonmotile (43) as is the well-characterized rabbit-pathogenic *E. coli* RDEC-1 (7). *Salmonella gallinarum*, a causative agent of fowl cholera, is, by definition, nonmotile (H^-) (30). *Vibrio cholerae* is a gram-negative enteric pathogen in which the role of motility in virulence appears ambiguous. Nonmotile derivatives of pathogenic vibrios were generally less adherent and less virulent than the parental strains (3, 16, 25, 41), but in comparisons between strains of different origins, the relationship between motility and virulence was variable (2, 5, 41). Pierce et al. (41) reported that motility was not associated with the immunogenicity of *V. cholerae*.

Many previous investigations of the role of *S. typhimurium* flagella have utilized in vitro assays of virulence (14, 19, 26, 48, 49). Under these conditions possession of active flagella appeared advantageous to bacteria, enabling a greater number of random collisions to occur between bacteria and mammalian cells, leading to irreversible attachment and invasion (26, 48). In accord with these observations, χ3419 (*mot-8008*::Tn*10*) and χ3422 (*fli-8007*::Tn*10*) demonstrated decreased invasiveness for tissue-culture cells in vitro compared with the wild-type strain SR-11 (Table 3).

The role of flagella as virulence factors in vivo is less certain. Carsiotis et al. (8) and Weinstein et al. (49) utilized isogenic strains of *S. typhimurium* each differing by a single genotypic defect. Wild-type, *motB*, and *flaF25* strains were compared in an in vitro assay for survival in macrophages and for virulence in mice. Their results indicate that flagella, whether active on the surface of wild-type motile strains or paralyzed on *motB* strains, were necessary for *S. typhimurium* to invade and cause severe disease. The nonflagellated strains were equally proficient at colonization of the murine intestinal tract, but these mutants were deficient in invasion of the reticuloendothelial system (49). Iino and co-workers originally characterized *flaF25* as a deletion of unknown size within the *flaF* gene cluster (23) which later was reported to encompass genes *flaFI* through *flaFV* (28). Carsiotis et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1987) have proposed that the deletion actually extends into a closely linked virulence gene(s) on the *S. typhimurium* chromosome. The attenuating aspects of the loss of flagella due to the introduction of *flaF25* in *S. typhimurium* have now been attributed to the deletion involving the previously uncharacterized virulence gene(s).

The mutations described in this report that abrogated the motility of *S. typhimurium* were the result of the stable integration of the transposon Tn*10* into genes responsible for either the biosynthesis of flagella or the motor activity of these organelles. The *fli-8007*::Tn*10* derivatives possessed no detectable flagella, and the *mot-8008*::Tn*10* mutants synthesized normal quantities of inactive filaments that were otherwise like those of wild-type *S. typhimurium* (Fig. 1 and 2). The results given in Table 3 show that in murine typhoid the *fli-8007*::Tn*10* mutation did not alter the virulence of strains SR-11 or SL1344, as the p.o. LD_{50}s of the *fli-8007*::Tn*10* mutants were virtually identical to those of the isogenic wild-type strains. The observable effects of the *mot-8008*::Tn*10* mutation depended upon the route of challenge. There were no statistically significant differences between the p.o. or i.p. LD_{50}s for the wild-type or nonmotile strains, though in mice challenged p.o., the LD_{50}s of the *mot-8008*::Tn*10* mutants were reproducibly increased by $\log_{10}$ 0.93 relative to the wild-type motile progenitor. If active flagella serve an unknown function in vivo, then a consequence of the *mot-8008*::Tn*10* mutation might come from the metabolic waste of energy used to synthesize paralyzed flagella. Biosynthesis of flagella imposes an expenditure of 2% of cellular energy during growth, while the flagellar motor requires only 0.1% of cellular energy (35). Nonetheless, there was no selection for loss of flagella, since χ3419 recovered from infected spleens was agglutinated by H antisera. Paralyzed cells did not demonstrate significant differences in rates of cell division, cell size (13), or ATP content (12) when compared with nonflagellated or wild-type cells.

The lack of attenuation of nonmotile *S. typhimurium*

strains was not restricted to specific *fla* mutations. Independent Tn*10* insertions that were mapped to different flagellar genes did not affect the virulence of *S. typhimurium* for mice.

The indifference of *S. typhimurium* to the presence or absence of active flagella on the cell surface is a measure of the high degree of virulence of this organism for BALB/c mice and suggests that motility might be irrelevant as a virulence factor for an invasive, facultatively intracellular pathogen. Uptake and dissemination of *S. typhi* and *S. typhimurium* by phagocytes is pathognomonic of typhoid fever in humans and inbred strains of mice, respectively (22). Motile *S. typhimurium* strains are immobilized by the viscocity of the mucus overlaying the gastrointestinal epithelium (38), and after intracellular invasion has occurred, disseminated infection results from the migration of infected macrophages through the host. The results reported here indicate that flagella and motility played a role in the ability of *S. typhimurium* to infect tissue culture monolayers in vitro, but flagella were not a virulence factor in the pathogenesis of murine typhoid. The *mot-8008*::Tn*10* mutation may be disadvantageous to bacteria in vivo, but the unaltered virulence of *fli-8007*::Tn*10* mutants in mice presents a critical test of the contribution of flagella to *S. typhimurium* virulence.

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LITERATURE CITED

1. **Ames, G. F.-L., E. N. Spudich, and H. Nikaido.** 1974. Protein composition of the outer membrane of *Salmonella typhimurium*: effect of lipopolysaccharide mutations. J. Bacteriol. **117**:406–416.
2. **Attridge, S. R., and D. Rowley.** 1983. The role of the flagellum in the adherence of *Vibrio cholerae*. J. Infect. Dis. **147**:864–872.
3. **Baselski, V. S., S. Upchurch, and C. D. Parker.** 1978. Isolation and phenotypic characterization of virulence-deficient mutants of *Vibrio cholerae*. Infect. Immun. **22**:181–188.
4. **Belas, M. R., and R. R. Colwell.** 1982. Adsorption kinetics of laterally and polarly flagellated *Vibrio*. J. Bacteriol. **151**:1568–1580.
5. **Bhattacharjee, J. W., and B. S. Srivastava.** 1979. Adherence of wild-type and mutant strains of *Vibrio cholerae* to normal and immune intestinal tissue. Bull. W.H.O. **57**:123–128.
6. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
7. **Cantey, J. R., and R. K. Blake.** 1977. Diarrhea due to *Escherichia coli* in the rabbit: a novel mechanism. J. Infect. Dis. **135**:454–462.
8. **Carsiotis, M., D. L. Weinstein, H. Karch, I. A. Holder, and A. D. O'Brien.** 1984. Flagella of *Salmonella typhimurium* are a virulence factor in infected C57BL/6J mice. Infect. Immun. **46**:814–818.
9. **Curtiss, R., III.** 1965. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. J. Bacteriol. **89**:28–40.
10. **Curtiss, R., III, S. M. Kelly, P. A. Gulig, C. R. Gentry-Weeks, and J. E. Galán.** 1988. Avirulent salmonellae expressing virulence antigens from other pathogens for use as orally administered vaccines, p. 311–328. *In* J. A. Roth (ed.), Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C.
11. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Enomoto, M.** 1966. Genetic studies of paralyzed mutants in *Salmonella*. I. Genetic fine structure of the *mot* loci in *Salmonella typhimurium*. Genetics **54**:715–726.
13. **Enomoto, M., and T. Iino.** 1963. Colonial dimorphism in nonmotile *Salmonella*. J. Bacteriol. **86**:473–477.
14. **Fields, P. I., R. V. Swanson, C. D. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83**:5189–5193.
15. **Galán, J. E., and R. Curtiss III.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86**:6383–6387.
16. **Guentzel, M. N., and L. J. Berry.** 1975. Motility as a virulence factor for *Vibrio cholerae*. Infect. Immun. **11**:890–897.
17. **Gulig, P. A., and R. Curtiss III.** 1987. Plasmid-associated virulence of *Salmonella typhimurium*. Infect. Immun. **55**:2891–2901.
18. **Hackett, J., S. Attridge, and D. Rowley.** 1988. Oral immunization with live, avirulent *fla*$^+$ strains of *Salmonella* protects mice against subsequent oral challenge with *Salmonella typhimurium*. J. Infect. Dis. **157**:78–84.
19. **Hohmann, A. W., G. Schmidt, and D. Rowley.** 1978. Intestinal colonization and virulence of *Salmonella* in mice. Infect. Immun. **22**:763–770.
20. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291**:238–239.
21. **Homma, M., K. Kutsukake, T. Iino, and S. Yamaguchi.** 1984. Hook-associated proteins essential for flagellar filament formation in *Salmonella typhimurium*. J. Bacteriol. **157**:100–108.
22. **Hook, E. W.** 1985. *Salmonella* species (including typhoid fever), p. 1256–1269. *In* G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett (ed.), Principles and practice of infectious diseases, 2nd ed. J. Wiley & Sons, Inc., New York.
23. **Iino, T., and M. Enomoto.** 1966. Genetical studies of nonflagellate mutants of *Salmonella*. J. Gen. Microbiol. **43**:315–327.
24. **Iino, T., Y. Komeda, K. Kutsukake, R. M. Macnab, P. Matsumura, J. S. Parkinson, M. I. Simon, and S. Yamaguchi.** 1988. New unified nomenclature for the flagellar genes of *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **52**:533–535.
25. **Jones, G. W., and R. Freter.** 1976. Adhesive properties of *Vibrio cholerae*: nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. Infect. Immun. **14**:240–245.
26. **Jones, G. W., L. A. Richardson, and D. Uhlman.** 1981. The invasion of HeLa cells by *Salmonella typhimurium*: reversible and irreversible bacterial attachment and the role of bacterial motility. J. Gen. Microbiol. **127**:351–360.
27. **Kagawa, H., T. Nishiyama, and S. Yamaguchi.** 1983. Motility development of *Salmonella typhimurium* cells with *flaV* mutations after addition of exogenous flagellin. J. Bacteriol. **155**:435–437.
28. **Kutsukake, K., T. Iino, Y. Komeda, and S. Yamaguchi.** 1980. Functional homology of *fla* genes between *Salmonella typhimurium* and *Escherichia coli*. Mol. Gen. Genet. **178**:59–67.
29. **Kutsukake, K., Y. Ohya, S. Yamaguchi, and T. Iino.** 1988. Operon structure of flagellar genes in *Salmonella typhimurium*. Mol. Gen. Genet. **214**:11–15.
30. **Le Minor, L.** 1984. Genus III. *Salmonella* Lignières 1900, 389^{AL}, p. 427–458. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins Co., Baltimore.
31. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
32. **Liu, S.-L., T. Ezaki, H. Miura, K. Matsui, and E. Yabuuchi.** 1988. Intact motility as a *Salmonella typhi* invasion-related

factor. Infect. Immun. **56**:1967–1973.
33. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the 'major outer membrane protein' of *Escherichia coli* K12 into four bands. FEBS Lett. **58**:254–258.
34. **Mackenzie, G. M., H. Fitzgerald, and R. Pike.** 1935. Interrelationships of antigenic structure, virulence and immunizing properties of smooth and rough cultures of *Salmonella aertrycke*. Trans. Assoc. Am. Physicians **50**:242–248.
35. **Macnab, R. M.** 1987. Motility and chemotaxis, p. 732–759. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Maganasik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
36. **Macnab, R. M., and S.-I. Aizawa.** 1984. Bacterial motility and the bacterial flagellar motor. Annu. Rev. Biophys. Bioeng. **13**:51–83.
37. **Mayfield, C. I., and W. E. Inniss.** 1977. A rapid, simple method for staining bacterial flagella. Can. J. Microbiol. **23**:1311–1313.
38. **McCormick, B. A., B. A. D. Stocker, D. C. Laux, and P. S. Cohen.** 1988. Roles of motility, chemotaxis, and penetration through and growth in intestinal mucus in the ability of an avirulent strain of *Salmonella typhimurium* to colonize the large intestine of streptomycin-treated mice. Infect. Immun. **56**:2209–2217.
39. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537. (Erratum, **129**:1171, 1977.)
40. **Meynell, E. W.** 1961. A phage, $\phi\chi$, which attacks motile bacteria. J. Gen. Microbiol. **25**:253–290.
41. **Pierce, N. F., W. C. Cray, Jr., J. B. Kaper, and J. J. Mekalanos.** 1988. Determinants of immunogenicity and mechanisms of protection by virulent and mutant *Vibrio cholerae* O1 in rabbits. Infect. Immun. **56**:142–148.
42. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493–497.
43. **Rowe, B., and R. J. Gross.** 1984. Genus II. *Shigella* Castellani and Chalmers 1919, 936^{AL}, p. 423–427. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins Co., Baltimore.
44. **Schmieger, H.** 1972. Phage P22-mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119**:75–88.
45. **Schneider, H. A., and N. D. Zinder.** 1956. Nutrition of the host and natural resistance to infection. V. An improved assay employing genetic markers in the double strain inoculation test. J. Exp. Med. **103**:207–223.
46. **Silverman, M., and M. I. Simon.** 1977. Bacterial flagella. Annu. Rev. Microbiol. **31**:397–419.
47. **Smyth, C. J.** 1988. Assays for fimbrial adhesins, p. 223–244. *In* P. Owen and T. J. Foster (ed.), Immunochemical and molecular genetic analysis of bacterial pathogens. Elsevier Science Publishing, Inc., New York.
48. **Tomita, T., and S. Kanegasaki.** 1982. Enhanced phagocytic response of macrophages to bacteria by physical impact caused by bacterial motility or centrifugation. Infect. Immun. **38**: 865–870.
49. **Weinstein, D. L., M. Carsiotis, C. R. Lissner, and A. D. O'Brien.** 1984. Flagella help *Salmonella typhimurium* survive within murine macrophages. Infect. Immun. **46**:819–825.
50. **Yamaguchi, S., H. Fujita, A. Ishihara, S.-I. Aizawa, and R. M. Macnab.** 1986. Subdivision of flagellar genes of *Salmonella typhimurium* into regions responsible for assembly, rotation, and switching. J. Bacteriol. **166**:187–193.
51. **Zinder, N. D., and J. Lederberg.** 1952. Genetic exchange in *Salmonella*. J. Bacteriol. **64**:679–699.